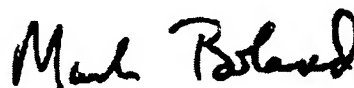


**REMARKS**

Entry and consideration of this Amendment are respectfully requested.

Respectfully submitted,



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**PATENT APPLICATION**  
**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of

Docket No: Q96337

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For: FEED ADDITIVE FOR LAYING HEN AND FEED CONTAINING THE SAME

# Substitute Specification with Marked Changes

## Description

FEED ADDITIVE FOR LAYING HEN AND FEED CONTAINING THE SAME

## 5 Technical Field

[0001]

This invention relates to a feed additive for laying  
hens and a feed containing the same. More specifically, it  
relates to a feed additive having an effect of reinforcing  
10 eggshells and a feed containing the same.

## Background Art

[0002]

Bird eggs such as chicken eggs are highly nutritious  
15 foods containing various nutritional components. Moreover,  
eggs are highly suitable for cooking. Owing to these  
characteristics, eggs are very important food resources.  
In the process of delivering newly laid eggs to consumers,  
however, eggshells are sometimes broken (egg breakage) or  
20 finely cracked (egg cracking). These broken eggs and  
cracked eggs have little or no commercial value, which  
causes an increase in the cost. Owing to the introduction  
of automatic detectors, the rate of eliminating cracked  
eggs, which used to range from 7 to 8%, attains 12 to 15%  
25 in these days and puts serious pressure on egg farmers'  
profits.

[0003]

It is considered that egg breakage and egg cracking arise since eggshells are weakened because of stress or failure in calcium utilization and so on. Calcium participating in eggshell formation is mainly supplied by feeds. Therefore, it is well known that calcium insufficiency in a feed results in thinning and weakening in eggshells.

[0004]

To overcome the problems of egg breakage and egg cracking, there have been employed methods of 1) shortening the egg collection period; 2) forced molting (stop laying by fasting); and 3) administering an eggshell reinforcing agent.

[0005]

Examples of the eggshell reinforcing agent as described above in 3) include beef meat-and-bone meal serving as a calcium source, calcium carbonate, CPP (casein phosphopeptide) capable of increasing the absorptivity of calcium (see Patent Document 1), an eggshell reinforcing agent comprising crab shell for reinforcing an eggshell per se together with chitosan, etc. for strengthening the eggshell membrane supporting the eggshell (see Patent Document 2). In addition, there have been known a feed containing poly- $\gamma$ -glutamic acid (see Patent Document 3) and

an eggshell reinforcing agent comprising trehalose (see Patent Document 4).

[0006]

On the other hand, lactobionic acid represented by O-  
5  $\beta$ -D-galactopyranosyl-(1-4)-D-gluconic acid has been already  
approved by FDA and added to pudding premixes as a  
solidifying agent or employed in cosmetics as a humidifier  
in the United States. Concerning functions, it has been  
reported that lactobionic acid shows activities of  
10 selectively stimulate the proliferation of bifid bacteria  
(see Patent Document 5) and stimulating mineral absorption  
(see Patent Document 6). It has been also reported that  
the administration of a chelate composed of lactobionic  
acid, iron and copper resulted in an increase in the  
15 hemoglobin level and improvement in anemia (see Non-patent  
Document 1). However, it has never been known that  
lactobionic acid has an effect of reinforcing eggshells.

[0007]

Patent Document 1: JP-A-1-285158

20 Patent Document 2: JP-A-9-47232

Patent Document 3: JP-A-9-28309

Patent Document 4: JP-A-2001-95499

Patent Document 5: JP-A-7-277990

Patent Document 6: JP-A-7-277991

25 Non-patent Document 1: The American Journal of  
Clinical Nutrition, 1982, Vol.36, ~~No.6,~~ p.1162 to 1169

## Disclosure of the Invention

Problems that the Invention is to Solve

[0008]

5           However, the forced molting 2) suffers from a problem from the viewpoint of animal protection. Moreover, it is reported that forced molting induces salmonella infection, which brings about another problem of worsening safety of chicken eggs. Furthermore, there arises an additional  
10 problem that the egg-laying rate is lowered due to stress caused by forced molting, cage transfer, etc.

[0009]

          The existing eggshell reinforcing agents 3) suffer from a problem that their effects would lower in the second  
15 half of the laying period.

[0010]

          An object of the present invention is to provide a feed additive for laying hens, which is excellent in effect of reinforcing eggshells and expected as showing a  
20 particularly remarkable effect of improving eggshell qualities in the second half of the laying period, relieves stress caused by forced molting, cage transfer and so on and can inhibit lowering in the egg-laying rate.

## Means for Solving the Problems

[0011]

To achieve the above-described object, the inventors conducted intensive studies. As a result, they have found  
5 out that lactobionic acid, which is represented by a general formula  $O-\beta\text{-D-galactopyranosyl-(1-4)-D-gluconic}$  acid, or its salt can remarkably reinforce eggshells, thereby completing the present invention.

[0012]

10 Accordingly, the gist of the invention resides in a feed additive for laying hens which comprises lactobionic acid or its salt. In another embodiment, the gist of the invention resides in a feed characterized by containing a feed additive for laying hens comprising lactobionic acid  
15 or its salt.

## Effect of the Invention

[0013]

According to the present invention, eggshells of  
20 chicken eggs and so on can be reinforced and thus the egg breakage rate and the egg cracking rate can be lowered. It is also possible to lessen fecal odor of chickens etc. Moreover, it is possible to relieve stress due to cage transfer, forced molting, etc. and improve the egg-laying  
25 rate, the egg qualities and the eggshell qualities.

## Brief Description of Drawing

[0014]

[Fig. 1] Fig. 1 shows changes in egg-laying rate with the passage of time.

5

## Best Mode for Carrying Out the Invention

[0015]

Now, the present invention will be described in greater detail.

10 [0016]

Lactobionic acid to be used in the present invention, which is represented by a general formula  $O-\beta-D$ -galactopyranosyl-(1-4)-D-gluconic acid, can be obtained by oxidizing lactose. More specifically, it can be obtained by treating lactose as the substrate with a microorganism having a lactose dehydrogenase activity such as *Pseudomonas graveolens* or oxidizing lactose with bromine or the like. From the economical viewpoint, it is most advantageous to employ a method which comprises oxidizing lactose by oxidase acting on lactose as the substrate or a microorganism having this enzyme which belongs to the genus *Acinetobacter* or *Burkholderia* (see, in detail, JP-A-2001-245657).

[0007]

25 The lactobionic acid acids usable in the invention include calcium salt, sodium salt, potassium salt,



magnesium salt, iron salt, zinc salt, copper salt and so on. These salts can be prepared by conventional reaction methods for converting into salt such as neutralization. Among them, calcium salt can be also prepared by a method which comprises preliminarily adding calcium carbonate to a reaction system for producing lactobionic acid from lactose by a biological conversion method with the use of an enzyme or a microorganism. This method is more convenient.

[0018]

In general, calcium cannot be absorbed unless it is in the soluble state in the intestinal tract. Because of having an extremely high solubility in water ( $>40$  g/100 mL), calcium lactobionate is excellent in the calcium-supplying effect and usable as a calcium source for eggshells even in the absence of any other calcium sources.

[0019]

Considering convenience in using, the feed additive for laying hens according to the invention, which comprises lactobionic acid or its salt as described above, can be obtained by processing them into a powder, an aqueous solution or compressed tablets to give a feed additive for laying hens.

[0020]

By administering the feed additive for laying hens to chickens either directly or as a mixture with a feed for chickens or drinking water, it is possible to reinforce

eggshells and lower the egg breakage rate and the egg cracking rate compared with the case of not administering the same. The feed additive for laying hens according to the invention may be administered to any birds without  
5 restriction. Thus, it is usable for quails, ducks and so on in addition to domestic fowl. That is to say, the term "laying hens" as used in the present invention involves quails, ducks and so on.

[0021]

10           The feed according to the invention can be obtained by adding the feed additive for laying hens as described above to a basic feed.

[0022]

          The basic feed for chickens may be an arbitrary one  
15 commonly employed for a feed for chickens. For example, use can be made of those obtained by blending organic nutritional sources such as corn, milo, soybean meal, fish meal and wheat flour and inorganic nutritional sources such as sodium chloride, calcium phosphate and calcium carbonate  
20 with feed additives comprising trace elements such as vitamins, metals and antibiotics.

[0023]

          The feed additive for laying hens is added to the basic feed preferably in an amount of from 0.01 to 10% by  
25 weight, more preferably from 0.05 to 1% by weight and most desirably from 0.1 to 0.5% by weight. When the addition

level is too low, it frequently takes a long time to achieve the desired effects. When the addition level is too high, there sometimes arise problems in nutritional balance or cost. In the case of adding lactobionic acid as the feed additive for laying hens, it is preferred to simultaneously add a calcium source such as calcium phosphate or calcium carbonate. In the case of adding calcium lactobionate as the feed additive for laying hens, it is not always necessary to add another calcium source.

10 [0024]

The feed additive for laying hens may be added to a feed by any method without particular restriction. For example, use may be made of a method of adding as a powder as such, a method of adding as an aqueous solution, a method of adding as compressed tablets and so on. Similarly, no restriction is imposed on feed form (powder, solid), feeding method or feeding time (day age of laying hens).

[0025]

20 It is preferable that the feed according to the invention is administered to laying hens in an amount of from 10 mg to 10 g per day in terms of the feed additive for laying hens contained therein, more preferably from 50 mg to 1.0 g and most desirably from 0.1 g to 0.5 g.

[0026]

It has been also clarified that by administering the feed additive for laying hens to chickens either directly or as a mixture with a feed for chickens or drinking water, a secondary effect of considerably lessening the fecal odor can be achieved compared with the case of not administering the same. Fecal odor emitted from chicken farms is a serious problem at present. According to the Law on Promoting Proper Management and Use of Livestock Excreta, feces should be contained in a roofed warehouse provided with a cement floor and dried before shipping from the end of March, 2004 in Japan. Therefore, it seems urgently required to develop an agent for lessening fecal odor.

[0027]

It is estimated that the effect of lessening fecal odor of the feed additive for laying hens according to the invention is caused by the function of lactobionic acid or its salt of controlling intestinal movements.

Examples

[0028]

Now, the present invention will be illustrated in greater detail by referring the following Examples. However, it is to be understood that the invention is not restricted thereto.

[0029]

[Example 1]

[Preparation of calcium lactobionate]

In 5 L of a 10 mM phosphate buffer solution (pH 7.0)  
5 containing 1000 g of lactose and 825 g of  $\text{CaCO}_3$ , 200 g of  
GRINGAMYL SURE BAKE 800 (manufactured by DANISCO, a  
recombinant enzyme of marine algae-origin hexose oxidase)  
was suspended to give a liquid reaction mixture. This  
liquid reaction mixture was allowed to react under stirring  
10 and aerating (blowing air at a rate of 0.5 times by volume  
as much as the liquid reaction mixture per minute) for 2  
days at 30°C. Then, the liquid reaction mixture was  
centrifuged (10,000 rpm, 30 minutes at 4°C) to thereby  
remove  $\text{CaCO}_3$ . The obtained supernatant was supplied into  
15 an active carbon column (5 cm (diameter) x 40 cm) having  
been equilibrated with deionized water and eluted with 1500  
ml of deionized water. The eluate was collected,  
concentrated to 300 mL under reduced pressure and then  
precipitated by adding ethanol 2.3 times as much. The  
20 precipitate was dried under reduced pressure to thereby  
give 1020 g of calcium lactobionate.

[0030]

As the results of HPLC analysis (column: Asahipak  
 $\text{NH}_2\text{P}-50$  (manufactured by Shodex), eluent: acetonitrile/40  
25 mM citric acid- $\text{NaH}_2\text{PO}_4$  buffer (pH 5.0)=60/40 (ratio by  
volume), temperature: 40°C, flow rate: 0.8 ml/min,

detector: differential refractometer (Waters 410:  
manufactured by Waters)) on the lactobionic acid, no  
elution peak assignable to impurities was found out, which  
indicated that the obtained product was highly pure

5 lactobionic acid. The solubility in water of the calcium  
lactobionate thus obtained was compared with those of other  
calcium salts (manufactured by Wako Pure Chemical  
Industries). Table 1 shows the results. Thus, it can be  
understood that calcium lactobionate has a much higher  
10 solubility in water than other calcium salts.

[0031]

[Table 1]

Solubilities in water of various calcium salts (g/100 mL)

Ca oxalate	0.001	Ca diphosphate	0.03	Ca gluconate	3.8
Ca carbonate	0.0014	Ca citrate	0.96	Ca lactobionate	>40

15 [0032]

[Examples 2 to 5]

[Preparation of feed]

To a basic feed for laying hens having the  
composition as listed in Table 2, calcium lactobionate  
20 prepared in Example 1 was added at concentrations of 0.05,  
0.1, 1.0 and 2.0% by weight to give feeds which were  
referred to respectively as Examples 2 to 5.

[0033]

[Table 2]

Composition of basic feed (%)

Starting material	Content (%)
Yellow corn	69.4
Soybean meal	16.0
CP65% fish meal	3.0
Alfalfa meal	2.0
DL-Methionine	0.1
L-Lysine hydrochloride	0.1
Calcium carbonate	6.5
Di-lime phosphate	2.0
Sodium chloride	0.3
Premix of inorganic trace elements <sup>1)</sup>	0.2
Premix of vitamins A, D and E <sup>2)</sup>	0.2
Premix of vitamin B family <sup>3)</sup>	0.2
Total	100

1) Containing 80 g of Mn, 50 g of Zn, 6 g of Fe, 0.6  
5 g of Cu and 1 g of I per kg.

2) Containing 10,000 IU of vitamin A, 2,000 IU of  
vitamin D<sub>3</sub> and 20 mg of vitamin E per g.

3) containing 2.0 g of thiamine nitrate, 10.0 g of  
riboflavin, 2.0 of pyridoxine hydrochloride, 2.0 g of  
10 nicotinamide, 4.35 g of calcium D-pantothenate, 138.0 g  
choline chloride and 1.0 g of folic acid per kg.

[0034]

[Test Example 1]

Ten laying hens aged 470 days (laying white leghorn  
15 hens (Julia)) were fed with each of the feeds prepared in  
Examples 2 to 5 for 60 days and the egg-laying rate, the  
egg breakage and egg cracking rate and eggshell strength

were measured. The egg-laying rate was determined in accordance with the following Equation 1, while the egg breakage and egg cracking rate were determined in accordance with the following Equation 2. Eggshell strength was determined by horizontally placing an egg and measuring the bursting strength with a rheometer. A feed containing no feed additive for laying hens was also prepared as a control. During the test period, no chicken died. Table 3 summarizes the results.

10 [0035]

[Equation 1]

Egg-laying rate =

$$\frac{\text{(total number of eggs in each test lot/}}{\text{total number of eggs in control lot)} \times 100$$

15 [0036]

[Equation 2]

Egg breakage/cracking rate =

$$\frac{\text{(number of broken or cracked eggs/}}{\text{total number of eggs yield)} \times 100$$



[0037]

[Table 3]

Test lot	Addition level (%) of feed additive	Egg-laying rate (%)	Egg breakage/cracking rate (%)	Eggshell strength (kg/cm <sup>2</sup> )
Control	0	100	7.3	2.8
Ex. 2	0.05	100.3	5.0	3.2
Ex. 3	0.1	99.1	3.2	3.8
Ex. 4	1.0	100.5	2.0	3.9
Ex. 5	2.0	101.0	1.8	4.1

[0038]

5           As the results given in Table 3 indicate, each of the test lots of Examples 2 to 5 showed no difference in the egg-laying rate from the control lot but an obvious decrease in the egg breakage and egg cracking rate and an improvement in the eggshell strength. Each of the test  
10 lots of Examples 2 to 5 showed no difference in the egg weight from the control lot. In each of the test lots of Examples 2 to 5, fecal odor was lessened and this tendency was particularly remarkable in the test lots of Examples 4 and 5.

15 [0039]

[Examples 6 to 9]

[Preparation of feed]

          To the basic feed for laying hens having the composition as shown in Table 2, lactobionic acid  
20 (manufactured by Wako Pure Chemical Industries) was added at concentrations of 0.05, 0.1, 1.0 and 2.0% by weight to

give feeds which were referred to respectively as Examples 6 to 9.

[0040]

[Test Example 2]

5           Ten laying hens aged 280 days (laying white leghorn hens (Julia)) were fed with each of the feeds prepared in Examples 6 to 9 for 70 days and the egg-laying rate, the egg breakage and egg cracking rate and eggshell strength were measured in the same manner as in Examples 2 to 5.

10          During the test period, no chicken died. Table 4 summarizes the results.

[0041]

[Table 4]

Test lot	Addition level (%) of feed additive	Egg-laying rate (%)	Egg breakage/cracking rate (%)	Eggshell strength (kg/cm <sup>2</sup> )
Control	0	100	5.0	3.2
Ex. 6	0.05	99.6	3.4	3.8
Ex. 7	0.1	101.2	2.2	3.8
Ex. 8	1.0	99.2	2.0	4.0
Ex. 9	2.0	100.7	2.3	4.1

15          [0042]

As the results given in Table 4 indicate, each of the test lots of Examples 6 to 9 showed no difference in the egg-laying rate from the control lot but an obvious decrease in the egg breakage and egg cracking rate and an  
20          improvement in the eggshell strength. Each of the test

lots of Examples 6 to 9 showed no difference in the egg weight from the control lot. In each of the test lots of Examples 6 to 9, fecal odor was lessened and this tendency was particularly remarkable in the test lots of Examples 8 and 9.

[0043]

[Examples 10 and 11]

From the composition of the basic feed for laying hens as shown in Table 2, calcium carbonate and di-lime phosphate were removed. To the obtained feed, calcium lactobionate prepared in Example 1 was added at concentrations of 1.0 and 2.0% by weight to give feeds which were referred to respectively as Examples 10 and 11.

[0044]

[Test Example 3]

Ten laying hens aged 470 days (laying white leghorn hens (Julia)) were fed with each of the feeds prepared in Examples 10 and 11 for 60 days and the egg-laying rate, the egg breakage and egg cracking rate and eggshell strength were measured in the same manner as in Examples 2 to 5. During the test period, no chicken died. Table 5 summarizes the results.

[0045]

[Table 5]

Test lot	Addition level (%) of feed additive	Egg-laying rate (%)	Egg breakage/cracking rate (%)	Eggshell strength (kg/cm <sup>2</sup> )
Control	0	100	7.3	2.8
Ex. 10	1.0	100.8	2.1	4.0
Ex. 11	2.0	100.5	1.9	4.1

[0046]

As the results given in Table 5 indicate, each of the  
5 test lots of Examples 10 and 11 showed no difference in the  
egg-laying rate from the control lot but an obvious  
decrease in the egg breakage and egg cracking rate and an  
improvement in the eggshell strength. Accordingly, it was  
clarified that calcium lactobionate alone showed an effect  
10 of reinforcing eggshells even in the case of adding no  
calcium source except calcium lactobionate to feeds. Each  
of the test lots of Examples 10 and 11 showed no difference  
in the egg weight from the control lot. In Examples 9 and  
10, fecal odor was remarkably lessened.

15 [0047]

[Examples 12 to 14] [Preparation of feed]

To the basic feed for laying hens having the  
composition as shown in Table 2, calcium lactobionate was  
added at concentrations of 0.1, 0.5 and 1.0% by weight to  
20 give feeds which were referred to respectively as Examples  
12 to 14.

[0048]

[Test Example 4]

Four laying hens aged 500 days (Boris Brown) were fed with each of the feeds prepared in Examples 12 to 14 for 60 days and the egg-laying rate and the eggshell strength were measured in the same manner as in Examples 2 to 5. During the test period, no chicken died. Table 6 summarizes the results.

[0049]

10 [Table 6]

Test lot	Addition level (%) of feed additive	Egg-laying rate (%)	Eggshell strength (kg/cm <sup>2</sup> )
Control	0	100	2.09
Ex. 12	0.1	113	2.36
Ex. 13	0.5	112	2.28
Ex. 14	1.0	103	2.53

[0050]

As the results given in Table 6 indicate, each of the test lots of Examples 12 to 14 showed an obvious improvement in the eggshell strength. Thus, it was clarified that an eggshell reinforcing effect could be obtained by adding calcium lactobionate to a feed. Each of the test lots of Examples 12 to 14 showed no difference in the egg weight from the control lot.

[0051]

(Test Example 5)

Four laying hens aged 500 days (Boris Brown) were fed with each of the feeds prepared in Examples 12 to 14 for 9 weeks. Feces of the laying hens were collected every week. The feces were suspended in 10 times as much distilled water and acetic acid contained in the supernatant was quantified by HPLC. Namely, the samples were analyzed by HPLC conducted under the following conditions: column: Aminex HPX 87H (300 x 7.8 mm (diameter), manufactured by BIORAD), column temperature: 60°C, developing solvent: 0.005 N sulfuric acid, sample amount: 30 µl, flow rate: 0.6 ml/min, detector: RI. Table 7 summarizes the results.

[0052]

15 [Table 7]

Amount of acetic acid in feces

Lot	Addition level (%) of feed additive	Acetic acid content (µg/mg)
Control	0.0	1.12 ± 0.81
Ex. 12	0.1	1.93 ± 1.74
Ex. 13	0.5	1.41 ± 0.85
Ex. 14	1.0	2.42 ± 1.26

[0053]

As the results given in Table 7 indicate, the acetic acid content was increased in each of the test lots of Examples 12 to 14 compared with the control lot. This is seemingly because calcium lactobionate was metabolized by

intestinal bacteria and thus promoted the proliferation thereof to thereby enhance acetic acid production by the intestinal bacteria. It appears that the intestinal bacteria in the laying hens were increased by the administration of calcium lactobionate and thus the intestinal environment was improved, which resulted in the lessening in the fecal odor.

[0054]

(Test Example 6)

Four laying hens aged 500 days (Boris Brown) were fed with each of the feeds prepared in Examples 12 to 14 for 3 weeks after cage transfer. Then the egg-laying rates were calculated. Fig. 1 summarizes the results.

As the results given in Fig. 1 indicate, a lowering in egg-laying rate due to cage transfer was observed in the control lot, while each of the test lots of Examples 12 to 14 showed a higher egg-laying rate than the control lot. This is seemingly because the stress loaded on the laying hens was relieved by calcium lactobionate.

[0055]

While the present invention and specific modes for the embodiment thereof have been described in detail, it is obvious for those skilled in the art that various alterations and modifications can be made without departing from the spirit and scope of the invention.

The present application is based on Japanese Patent Application filed on February 6, 2004 (Japanese Patent Application No. 2004-030898) and the contents thereof are employed herein as reference.

5

#### Industrial Applicability

According to the present invention, it is possible to provide a feed additive for laying hens which is excellent in effect of reinforcing eggshells, shows a particularly  
10 remarkable effect of improving eggshell qualities in the second half of the laying period, relieves stress caused by forced molting, cage transfer and so on and can inhibit lowering in the egg-laying rate.